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Evidence on the presence of two distinct alkaline phosphatases in *Serratia marcescens*

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Abstract

Certain strains of *Serratia marcescens* synthesized two different types of alkaline phosphatase (APase), constitutive (CAPase) and inducible (IAPase) APases, in low phosphate medium. Synthesis of the IAPase was repressed in the presence of high phosphate. Purification and separation of these electrophoretically distinct APases was achieved by using fractional $(NH_4)_2SO_4$ precipitation, adsorption on a DEAE-cellulose column and elution of enzymes by a linear sodium chloride gradient. Starch gel electrophoresis of certain fractions revealed the separation of not only IAPase from CAPase but its separation into four distinct isozymes. CAPase gave maximum enzyme activity around pH 9.5, whereas for IAPase a broad range of enzyme activity was found between pH 8.5 and 10.5. Reversible inactivation at low pH occurred for IAPase but very little with CAPase. CAPase was more thermolabile than IAPase at 95°C. The two APases were found to be distinct in their kinetic as well as immunological properties, suggesting two distinct enzyme species. Crown copyright © 2000 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Alkaline phosphatase (APase, EC 3.1.3.1) is of ubiquitous occurrence in plant, animal and insect tissues as well as in microorganisms. Since the discovery of APase in *Escherichia coli* [1] this enzyme has been studied extensively. Levinthal and his associates have reported many important similarities between the APases from *E. coli* and *Serratia marcescens*. In each case, inorganic phosphate (Pi) acted as a repressor as well as inhibitor of enzyme activity and three closely related isozymes were formed in vivo and in vitro. Experiments with intergeneric crosses showed that the regulatory mechanism of *E. coli* would also function in *S. marcescens* [2]. We reported previously that certain strains of *S. marcescens* synthesized two electrophoretically distinct isozyme species, inducible (IAPase) and constitutive (CAPase) APases, under limiting inorganic phosphate growth conditions [3]. These investigations have revealed an entirely novel phenomenon – namely, that in some strains of *S. marcescens* the synthesis of CAPase occurred in the cell when the synthesis of IAPase was repressed. In this paper we report the purification and separation of two electrophoretically distinct APases of *S. marcescens* 211, and investigated some of their biochemical properties.

2. Materials and methods

2.1. Organism and growth conditions

S. marcescens 211 was cultivated in 68 l of low phosphate medium [4] supplemented with lactic acid, 10 ml l^{-1}, at 30°C for 20 h as a batch culture. The growth of the culture was stopped when cells started to produce dense pink pigment.

2.2. Analytical procedures

Enzyme activity, protein concentrations and specific activity, starch gel electrophoresis and staining for APase activity were carried out as described earlier [5]. Thermal stability of the APases was performed according to the method described [6] at 95°C. The effect of pH on the

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reversible inactivation of APases was determined as reported [7]. The effect of pH on the two types of APases was determined according to the method of Wilson et al. [8]. Immunodiffusion was carried out as described [5].

3. Results and discussion

3.1. Isolation and purification of APases

The bacterial suspension was harvested and APase activity was isolated by the butanol extraction procedure [5] and dialyzed against Tris-Mg^{2+} buffer (Tris 10 mM, $MgCl_2$ 5 mM, pH 8.0) for 48 h at 4°C. All the APase activity was sedimented with the bacterial cells after centrifugation, no cell-free APase activity was detected in the cell-free medium. Therefore, unlike *Pseudomonas aeruginosa* [9], *Bacillus subtilis* [10] and *Haloarcula marismortui* [11], APases of *S. marcescens* are not secreted into the growth medium during growth and are cell-bound [5]. All other purification steps were carried out at 4°C.

3.2. First fractional precipitation of APases

Dialysate was fractionally precipitated using various concentrations of $(NH_4)_2SO_4$. Based on the specific activity, 45–90% $(NH_4)_2SO_4$ saturation fractions were pooled and dialyzed against Tris-Mg^{2+} buffer for 48 h. After dialysis and centrifugation a large amount of precipitate was found and discarded.

3.3. DE-11 cellulose chromatography

$(NH_4)_2SO_4$ dialysate from the previous step was applied onto the DE-11 cellulose column and enzyme eluted with 0.4 M NaCl in Tris-Mg^{2+} buffer. Ninety-five percent of the enzyme activity was eluted between fractions 12 and 30. These fractions were pooled and dialyzed against Tris-Mg^{2+} buffer as described above.

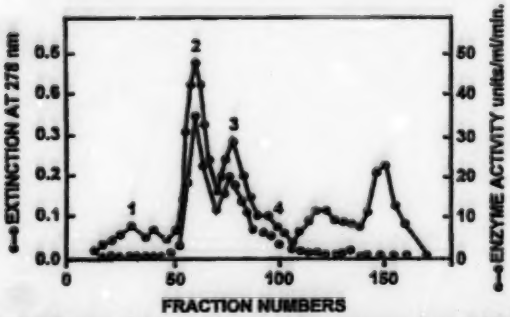

Fig. 1. Purification and separation of IAPase isozymes from CAPase of *S. marcescens* by DE-32 cellulose column chromatography. 273 mg of protein in 42 ml was applied to a DE-32 cellulose column (2.3×17 cm) that had been equilibrated with Tris-Mg^{2+} buffer. The column was washed with 30 ml of the same buffer. APases were eluted with a linear gradient of NaCl 0.00 mM (150 ml) to 0.05 mM (150 ml) in Tris-Mg^{2+} buffer. ○, enzyme activity; ●, extinction at 278 nm. Peaks 1–4 correspond to IAPase isozymes IV, III, II, and I respectively.

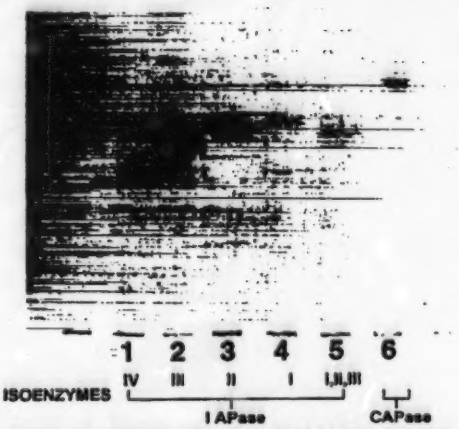

Fig. 2. Starch gel electrophoresis of purified IAPase isozymes and CAPase of *S. marcescens*. Samples from the pooled fractions containing separated isozymes after DE-32 cellulose chromatography were subjected to starch gel electrophoresis. A slice of starch gel was stained for protein, illustrated protein bands for individual isozymes IV, III, II, I and mixtures of isozymes I, II, and III of IAPase from different pooled fractions (lane 1, fractions 10–30; lane 2, fractions 56–67; lane 3, fractions 77–83; lane 4, fractions 98–106; lane 5, fractions 68–76,84–97) and lane 6, CAPase respectively. Faint protein bands for isozymes IV and I (lanes 1 and 5) seen in the gel after protein staining are not visible in the picture.

3.4. Second $(NH_4)_2SO_4$ fractional precipitation of APases

Dialysate from the previous step was fractionated with various concentrations of $(NH_4)_2SO_4$. High specific activity enzyme was precipitated from 40–70% $(NH_4)_2SO_4$ saturations. These fractions were pooled and dialyzed as in the previous step. Different fractions of $(NH_4)_2SO_4$ fractionation, when subjected to electrophoresis and stained for APase activity, did not result in the complete separation of IAPase from CAPase. However, it did indicate that the IAPase precipitated at 70% saturation was greater than the amount precipitated at 50% saturation.

3.5. DE-32 cellulose chromatography

Final purification and separation of APases was achieved by DE-32 cellulose column chromatography. Dialysate from the previous purification step was applied onto the DE-32 cellulose column. The APase activity was eluted using a linear gradient of NaCl in Tris-Mg^{2+} buffer. Fractions of 4 ml were collected, at a flow rate of 4 ml/5 min. These fractions were analyzed for UV absorption at 278 nm and APase activity. The results of the enzyme activity and UV absorption of various fractions are depicted in Fig. 1. These fractions, when analyzed by starch gel electrophoresis and stained for APase activity, demonstrated that IAPase isozymes were completely separated from one another but no CAPase activity was

eluted. The DE-32 cellulose column was then subjected to stepwise elution with various concentrations of NaCl. High specific activity of CAPase was eluted at 0.125 M NaCl. This showed that IAPase isozymes were eluted at lower NaCl (0.00–0.05 M NaCl) concentrations than CAPase. These results clearly demonstrate that the two APases are distinct in ionic strength required for elution from the DE-32 ion exchange column. Fractions containing individual isozymes were pooled and further analyzed electrophoretically. Results of starch gel electrophoresis showing the complete separations of these two enzyme species are illustrated in Fig. 2. IAPase isozymes are designated I, II, III and IV in order of their increasing mobility towards the anode [3]. Isozymes III, II and I (lanes 2, 3, 4) are clearly seen in Fig. 2. Faint protein bands for isozymes IV and I (lanes 1 and 5) seen in the gel after protein staining are not visible in the picture. Lane 6 shows one protein band for CAPase. Pooled fractions containing IAPase and CAPase along with rainbow molecular mass markers were subjected to SDS-PAGE according to Laemmli's method [12] in 10% acrylamide gel and stained with Coomassie blue. One major polypeptide band was seen for both APases at the 49- and 50-kDa range suggesting 98 and 100 kDA molecular masses for IAPase and CAPase respectively (Fig. 3). The degree of purification of these separated isozymes was very high, as Coomassie protein stain did not show any detectable contaminating protein. The results on the purification and separation of the two types of APases are summarized in Table 1.

3.6. Physical characteristics

These two APases were colorless and showed a typical UV absorption spectrum and gave single peaks at 279 and 278 nm for IAPase and CAPase respectively. The absence of any peak at 260 nm indicated the absence of significant contamination from nucleic acids (data not shown). These spectral properties are similar to those reported for a highly purified APase from *E. coli* [6] and *B. subtilis* [13].

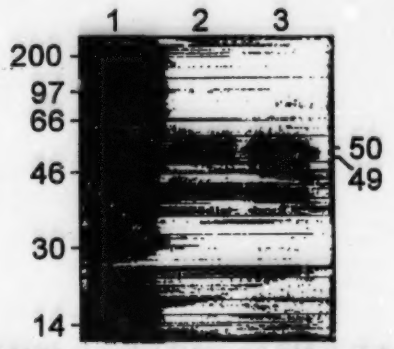

Fig. 3. SDS-PAGE of purified IAPase and CAPase of *S. marcescens*. Lane 1, rainbow molecular mass markers in kDa; 2, IAPase (sample 5 of Fig. 2); 3, CAPase (sample 6 of Fig. 2). The positions of the 50- and 49-kDa APases are as indicated.

3.7. Effect of pH and NaCl on the catalytic activities

CAPase gave maximum enzyme activity around pH 9.5, whereas for IAPase a broad range of enzyme activity was found between pH 8.5 and 10.5. However, the two enzymes behaved differently in 1 M NaCl. Distinct differences between the effect of 1 M NaCl on the two enzymes activities were found. The marked activation effect of 1 M NaCl on the IAPase activity contrasts with the inhibitory effect on CAPase activity (data not shown). The sharp decline observed above pH 9.5 for CAPase was a reversible pH-dependent change. This behavior, unlike APase from *B. subtilis* [10], is similar to APase from *E. coli* [14].

3.8. Reversible inactivation at low pH

The effects of H^+ ion concentration on *S. marcescens*

Table 1
Purification and separation of inducible and constitutive alkaline phosphatase isozymes of *S. marcescens*

Procedure	Volume (ml)	Total enzyme activity (units)[a]	Total protein (mg)	Specific activity	Yield (%)	Purification (fold)
Cell suspension	68 000	13 600	27 200	0.5	100	1.0
Butanol extract	1 190	13 700	2142	6.4	100	12.6
First $(NH_4)_2SO_4$ fractionation (40–90%)	190	8 820	689	9.2	46	18.4
DE-11 cellulose chromatography	240	6 270	528	9.8	38	19.6
Second $(NH_4)_2SO_4$ fractionation (40–70%)	42	5 160	273	32.3	65	64.6
DE-32 cellulose chromatography						
Inducible IAPase						
Isozyme IV	23	6.9	2.76	2.5	0.05	5.0
Isozyme III	30	426.0	19.20	22.2	3.13	44.4
Isozyme II	16	320.0	7.36	43.5	2.35	87.0
Isozyme I	20	88.0	2.86	30.8	0.65	61.8
Isozymes I,II,III	65	728.0	16.90	43.1	5.35	86.2
Constitutive CAPase	106	2014.0	19.08	106.0	14.81	212.0

[a]A unit of enzyme activity was defined as the quantity required to liberate 1 μmol of nitrophenol from *p*-nitrophenylphosphate per minute. The specific activity is expressed as units per mg protein.

Fig. 4. Immunological distinction of CAPase from IAPase. Antiserum to CAPase was applied in the central well. Surrounding wells: 1, IAPase; 2 and 3 CAPase.

APases show that CAPase and IAPase lost 99% and 93% of their initial enzyme activities after 2 min of incubation at pH 2.3, while 100% inactivation was observed after 10 min of incubation at 0°C. However, reactivation of these enzymes at 37°C in alkaline medium (pH 7.8) was different in each case. Unlike APase from *E. coli* [7], 85% of IAPase was reactivated after 60 min of incubation at 37°C and increased to 95% reactivation by 200 min. This is in sharp contrast to CAPase which remained largely inactive and only 5% could be reactivated by 200 min of incubation at 37°C (data not shown). In general, in APases from other sources [7,15–20], CAPase is a dimer of identical subunits [5], and the dimer formation is necessary for enzyme activity. The shape of the inactivation curve indicated that inactivation took place in two phases, an initial rapid inactivation followed by a slower reaction. This was consistent with earlier observations on the *E. coli* enzyme [7,15,16], the human intestine [17] and pig kidney APase [20]. Similar to CAPase, APase from *B. subtilis* [18] did not give active enzyme after acid treatment. Lowering of the pH to 2.3 probably caused a conformational change of CAPase to a structure that is no longer fully catalytically active as an enzyme [16,19]. If the first reaction of acid on CAPase was to induce a conformational change [18,20], then it is possible that the new conformation obtained at acid pH was more susceptible to acid denaturation than the former conformation. This would account for the impossibility of restoring 100% CAPase activity after acid treatment [20]. The reactivated APases had electrophoretic properties identical to those of non-treated enzymes (data not shown).

3.9. Thermal stability

These two APases also behaved distinctly in terms of their stability at high temperature. CAPase was found to be more thermolabile than IAPase at 95°C. Mg^{2+} ion, which seems to protect CAPase activity at 80°C [7], did not at 95°C. It lost all of its enzyme activity even in the presence of 10 mM Mg^{2+} after 5 min of incubation at 95°C. IAPase was relatively stable at 95°C. IAPase in the absence of Mg^{2+} lost 30 and 80% of its activity after 5 and 30 min of incubation at 95°C. Mg^{2+} ions, which protected *E. coli* APase [6], did not seem to protect IAPase activity at this temperature, the loss in its activity was found to be 20 and 75% after 5 and 30 min of incubation at 95°C (data not shown).

3.10. Kinetic parameters

The K_m values for *p*-nitrophenylphosphate as substrate were found to be 8.26×10^{-6} and 5.7×10^{-5} M for IAPase and CAPase respectively. Both APases were competitively inhibited by orthophosphate, the apparent K_i values being 5.3×10^{-6} and 7.6×10^{-4} M for IAPase and CAPase respectively. These two kinetic parameters also demonstrate that IAPase and CAPase of *S. marcescens* are two distinct enzyme activities.

3.11. Immunological identity

The antiserum prepared against CAPase [5,21], when tested by the Ochterlony double diffusion method, specifically reacted with its antigen but did not give any cross-reaction with IAPase (Fig. 4). It gave reaction of identity against purified CAPase obtained from *S. marcescens* 211 grown in high phosphate medium [5]. When subjected to immunoprecipitation assay, it precipitated CAPase activity completely, but no IAPase activity was precipitated. This indicates that the antigenic structures of IAPase and CAPase appeared different, and further suggests that these two proteins of *S. marcescens* are immunologically distinct enzyme species. Unlike the two types of APases reported from *B. subtilis* [10,18,22], the APases described in this study were distinct in their growth requirements, electrophoretic mobilities [3], chromatographic behavior, biochemical as well as immunological properties and therefore, were different isodynamic enzymes which probably arose from an independent genetic origin.

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